Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Role of *Pantoea agglomerans* in opportunistic bacterial seed and boll rot of cotton (*Gossypium hirsutum*) grown in the field

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Keywords
hard-lock(s), hollow seed, opportunistic infections, selection marker, stink bugs.

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Accession numbers: AY924374, AY924375 and AY924376.

2005/1178: received 5 October 2005, revised and accepted 13 April 2006

doi:10.1111/j.1365-2672.2006.03055.x

Abstract

Aims: To investigate the aetiology of seed and boll rot of cotton grown in South Carolina (SC).

Methods and Results: Bacteria were isolated from diseased locules of cotton bolls collected in a field in SC, USA and tested for the ability to cause comparable disease symptoms in greenhouse grown cotton fruit. Spontaneously generated rifampicin-resistant (Rif^r) mutants of the isolates were used in confirmatory pathogenicity tests. Resistance to the antibiotic was both stable and effective in differentiating between an inoculated Rif^r strain, rifampicin-sensitive contaminants and/or endophytes. A series of inoculation methods was tested at various boll developmental stages and at different fruiting nodes on the plant. Field disease symptoms were reproduced by inoculating bolls at 2 weeks postanthesis with bacterial suspensions ranging from 10^3 to 10^6 CFU ml^{-1}. Pathogenic isolates were categorized as *Pantoea agglomerans* on the basis of phenotype testing, fatty acid profiling (similarity index = 0·94), and 16s ribosomal DNA sequence analysis (99% nucleotide identity).

Conclusions: *Pantoea agglomerans* isolates from field-collected immature, diseased cotton caused comparable infection symptoms in greenhouse produced cotton fruit.

Significance and Impact of the Study: In 1999, significant yield losses in SC cotton resulted from a previously unobserved seed and boll rot that has since been reported in other southeastern states. This study demonstrated a role of *P. agglomerans* in producing opportunistic bacterial seed and boll rot of cotton.

Introduction

In recent years, seed and boll rot of South Carolina (SC) cotton (*Gossypium hirsutum*) has been reported to be responsible for losses of 10–15% of yield (Hudson 2000). This emerging disease of cotton has since been observed in fields located throughout the southeastern Cotton Belt, yet it primarily affects the SC harvest (Edmisten 1999; Hudson 2000). Initial attempts to identify a causal agent(s) of the disease were either unsuccessful or inconclusive (Hudson 2000; Hollis 2001). Early detection in affected fields is complicated by the absence of external infection symptoms on immature bolls. Disease symptoms include discolored lint and dead seed that can be observed only when infected immature bolls are cross-sectioned or when bolls open. Notably, immature diseased locules have been attributed to the development of so-called 'hard locks' that can occur in bolls also possessing asymptomatic locules (Hudson 2000). In contrast to a typically white and downy fibre, lint from infected locules (normally four locules per boll) is brown and dense. At harvest, open bolls with a hard lock(s) have either already dropped from the canopy or are not efficiently harvested resulting in economic loss.

A rudimentary disease paradigm for seed and boll rot of SC cotton includes: (i) asymptomatic outer carpels of infected bolls; (ii) incomplete maturity of fibre and seed; and (iii) brown necrotic coloration of both fibre and seed tissue. Mauney and Stewart (2003) described anatomical and developmental differences between embryos dissected from typical cotton seed and SC bolls exhibiting 'hollow seed', an idiom adopted pending the identification of a casual agent(s). They found that normal fertilization occurred in both situations (i.e. double fertilization resulting in the formation of an embryo and endosperm), yet a divergence in the subsequent growth was apparent. No difference in the ultimate seed size was observed; however, typical seed coat coloration occurred prematurely, and embryos from 'hollow seed' were smaller than embryos of healthy seed. In addition, seeds with the syndrome did not exhibit diagnostic fungal rot symptoms (Mauney *et al.* 2004).

In the current report, we discuss the isolation and identification of opportunistic *Pantoea agglomerans* from immature, diseased cotton bolls [Acala Maxxa cultivar (cv.)] that were collected from field plots in SC. Based on Koch's postulates, *P. agglomerans* strains were consistently isolated from diseased bolls and determined to be capable of producing disease symptoms in greenhouse grown cotton that closely resembled infected field boll samples. *Pantoea agglomerans* has traditionally been regarded as a soil saprophyte with a cosmopolitan distribution (Dye 1969). Strains of *P. agglomerans* have been recently utilized as an effective biological control agents due to production of antimicrobials and a capacity to competitively colonize plants (Wright *et al.* 2001; Costa *et al.* 2002; Stockwell *et al.* 2002; Poppe *et al.* 2003). Furthermore, opportunistic *P. agglomerans* infections are known to occur in both humans and plants. Opportunistic strains can cause septic arthritis in humans (Kratz *et al.* 2003), and galls on the ornamental plant gypsophila (*Gypsophila paniculata* L.) and on beets (*Beta vulgaris* L.) (Ezra *et al.* 2004).

Materials and methods

Bacterial isolation

Unopened cotton bolls grown from Acala Maxxa (cv.) seed were collected in August 2002 (i.e. late in the growing season) from field plots at the Pee Dee Research Center in SC and transported overnight on ice to the USDA-ARS Cotton Pathology Research Unit in College Station, Texas. Bolls were washed separately in a 0·5% sodium hypochlorite solution for 2 min and then rinsed three times with sterile water. Carpel walls were excised with a sterile scalpel and discarded. A flame-sterilized glass rod was rubbed on individual discolored seed and streaked on tryptic soy agar (TSA; Difco Laboratories, Detroit, MI, USA). Individual seeds were submerged and agitated in sterile water at 25°C and then placed on TSA; the supernatants were spread onto TSA. Controls consisted of bolls with asymptomatic seed which were processed as described for discolored seed samples. Plates were incubated at 25°C until colonies appeared but not longer than 2 weeks. Individual colonies were purified by dilution plating on TSA and stored at −80°C in a 40% glycerol solution diluted with 1% Luria Bertani (Difco Laboratories, Detroit, MI, USA).

Pathogenicity tests

Cotton plants (Acala Maxxa cv.) were grown in a greenhouse planting mixture that consisted of 18 l washed sand, 12 l vermiculite, 12 l dried peat moss, 1 l gypsum, 300 ml dolomitic lime and 50 ml esmigran (Scotts-Sierra Horticultural Products Co., Marysvillee, OH, USA). Plastic pots (0·5 kg) were filled with the mixture, saturated with reverse-osmosis water, and then pasteurized using aerated steam (74°C) for 16 h. Seedlings started in germination towels (48 h at 30°C) were transplanted into the planting mixture. Pathogenicity testing experiments using immature bolls were performed in glass greenhouses beginning March 2003. Cooling and heating thermostats were set at 30 and 20°C respectively. Weekly, plants received 150 mg Peter's Peat-Lite Special 15-16-17 containing chelated minor elements (Scotts-Sierra Horticultural Products Co., Marysvillee, OH, USA).

Bacterial isolates from separate diseased bolls were used in inoculation protocols for pathogenicity testing. Cultures were routinely maintained on TSA plates and grown at 28°C. The purity of the bacterial isolates was ensured by using individual colonies for each experiment. Bacterial suspensions in PO_4 buffer (0·1 mol l^{-1}, pH 7·1) were prepared from 18-h cultures on TSA and adjusted spectrophotometrically ($A_{600} = 1{\cdot}0$). A bacterial suspension at a final concentration of 10^8 CFU ml^{-1} of the isolate being tested was placed on stigma scars, wall sutures, and scratch wounds on bracts, calyx and bolls or a 28G needle was used to puncture through a drop placed on the boll wall suture, bracts or pedicel. Bolls were cross-sectioned 1, 2, 3 and 4 weeks postinoculation and observed for disease symptoms.

Resistance to rifampicin (Rif) was employed as a selection property to differentiate between spontaneously generated antibiotic-resistant mutants, susceptible contaminants and/or endophytes. The selection marker was incorporated into the genome of three of the suspected opportunistic pathogens (Sc1, Sc2 and Sc4) that were recovered from separate bolls and determined to be capable of producing infection symptoms in initial pathogen-

Table 1 Bacterial strains used in this study

Strains	Relevant characteristics	Source
Pantoea agglomerans		
ATCC 27155	Knee abrasion, type strain, Rif sensitive	American Type Culture Collection
Sc1	Diseased Acala Maxxa boll from SC field plot, Rif sensitive	This study
Sc2	Diseased Acala Maxxa boll from SC field plot, Rif sensitive	This study
Sc4	Diseased Acala Maxxa boll from SC field plot, Rif sensitive	This study
Sc1-R	Derived from Sc1, Rif resistant	This study
Sc2-R	Derived from Sc2, Rif resistant	This study
Sc4-R	Derived from Sc4, Rif resistant	This study
Escherichia coli K12		
ER2267	F′ proA$^+$B$^+$ lacI^{q}Δ(lacZ)M15 zzf::mini-Tn10 (Kan^R)/ Δ(argF-lacZ)U169 glnV44 e14$^-$ (McrA$^-$) rfbD1? recA1 relA1? endA1 spoT1? thi-1 Δ(mcrC-mrr)114::IS10	New England Biolabs, Inc.

Rif, rifampicin; SC, South Carolina.

icity tests (Table 1). Mutants with resistance to Rif were generated by incremental exposure of the isolates to M9 minimal medium (Sambrook *et al.* 1989) amended with Rif levels up to 200 μg ml^{-1}. Growth patterns of the mutants and the Rif-sensitive parental strains were compared by maintaining continuous cultures (shaken at 200 rpm) of the bacteria in tryptic soy broth at 28°C by transferring each to fresh media daily for 2 weeks. Every 2 days a subsample was dilution plated on both TSA and TSA amended with Rif (200 μg ml^{-1}), and colony counts were recorded following 24 h of incubation at 28°C.

The ability of the Rif-resistant mutants to cause infection was compared with the respective Rif-sensitive parental strains using bolls at a maturity of 13–15 days postanthesis (DPA). Two separate disease testing experiments were conducted with triplicate inoculations of a single locule per boll. Bacterial suspensions in PO_4 buffer (0·1 mol l^{-1}, pH 7·1) were prepared from 16-h cultures and adjusted spectrophotometrically ($A_{600} = 0{\cdot}5$). Bacterial suspensions were injected to a depth of 5 mm into the centre of the suture of a single locule per fruit at a final concentration of 10^6 CFU ml^{-1} using a 28G needle. Negative controls consisted of boll locules of the same maturity injected with 10 μl of PO_4 buffer. Two weeks following the inoculations, bolls were scored for the presence/absence of disease symptoms, and bacterial concentrations were determined. Bolls were washed separately in a 0·5% sodium hypochlorite solution for 10 min and then rinsed three times with sterile water. Carpel walls were excised with a sterile scalpel, aseptically removed, and discarded. One gram of seed and lint tissue from the inoculated locule was suspended in PO_4 buffer and triturated using a sterile mortar and pestle. All samples were 10-fold serially diluted and plated on TSA with and without Rif (200 μg ml^{-1}) and incubated at 28°C for 24 h. Bacterial colonies were enumerated and recorded as CFU g^{-1} locule tissue. In addition, bolls were allowed to mature (i.e. split and open), and the final disease symptoms were photographed.

The Rif-resistant mutant Sc1-R was used to study the impact of bacterial concentrations on disease symptom development and bacterial recovery from the boll locules. Bolls at a maturity ranging from 13 to 15 DPA were inoculated with final bacterial concentrations of 10^3, 10^6 or 10^8 CFU ml^{-1} using the protocols described above. Two separate disease testing experiments were conducted with inoculations performed in triplicate and included negative controls that consisted of bolls injected with heat-killed Sc 1-R cells (1 h at 65°C) at final concentrations equivalent to live cells or 10 μl of PO_4 buffer. Suspensions exposed to 65°C for 1 h were plated on TSA to confirm cell death. Two weeks following the inoculations, disease was scored based on the presence/absence of symptoms and bacterial densities (CFU g^{-1} locule tissue) were determined for both Sc1-R and control bolls by 10-fold serially diluting and plating on TSA with and without Rif (200 μg ml^{-1}).

Strain Sc1-R was used to determine the effect of bacterial inoculations into bolls at different maturity levels. Bolls were inoculated with a 10^6 CFU ml^{-1} final bacterial concentration (as described above) at 6–8, 13–15, 20–22 and 27–28 DPA. Two separate disease testing experiments were conducted with triplicate inoculations of a single locule per boll. The negative control consisted of locules of bolls of the same maturity injected with 10 μl of PO_4 buffer. Disease was scored based on the presence/absence of symptoms, and bacterial densities (CFU g^{-1} locule tissue) were determined.

Phenotypic characterization of pathogens

Representatives of the colony type consistently isolated from diseased field cotton samples and determined to produce analogous symptoms in greenhouse grown bolls

(Sc1, Sc2 and Sc4) were further characterized. The general strategy and methods used to identify the bacteria followed Schaad *et al.* (2001). The analysis consisted of a Gram reaction, anaerobic growth, catalase and oxidase production, and colony coloration on YDC media. The *P. agglomerans* type strain ATCC 27155 was used as a control in all tests. Upon putative classification of the representatives as Enterobacteriaceae, further tests were conducted to categorize the isolates to species. Analytical Profile Index (API; bioMerieux, Inc., Durham, NC, USA) 20E test strips were utilized for phenotypic characterization according to the manufacturer's instructions along with the control *P. agglomerans* type strain.

Fatty acid profiling and 16s ribosomal DNA sequencing

Isolates Sc1, Sc2 and Sc4 were submitted on a contract basis to the Texas Plant Disease Diagnostic Laboratory (TPDDL) at Texas A&M University, College Station, for independent identification. At the TPDDL, species identification and degree of similarity of the isolates was performed by comparing their fatty acid (FA) profiles to the MIDI library TSBA version 3.9 [http://plantpathology.tamu.edu/extension/tpddl/services.asp (accessed June 2006)].

A universal degenerate DNA polymerase chain reaction (PCR) primer set designed to generate a 16s ribosomal product (Jacobs *et al.* 2000) was modified by the addition of enzyme restriction sites (underlined) for cloning and sequencing (16sF*Xba*I – 5′-GGTCTAGAAGAGTTTGATCMTGGCTCAG-3′; 16sR*Not*I – 5′-CGGCGGCCGCACGGGCGGTGTGTACA-3′). The predicted 1·5-kbp fragment based on *Escherichia coli* positioning was ligated into the *Xba*I–*Not*I sites of the pDrive cloning vector (New England Biolabs Inc., Beverly, MA, USA) then transformed into *E. coli* ER2267 (Table 1) by the $CaCl_2$ method (Sambrook *et al.* 1989). Sequencing was performed at the Institute of Developmental and Molecular Biology, Gene Technologies Laboratory at Texas A&M University. Derived 16s rDNA gene sequence data from both strands were edited and assembled using Sequencher 4·2 (Gene Codes Corp., Ann Arbor, MI, USA) then compared with sequences in the GenBank database using the BLAST program offered by the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/; accessed June 2006). A Qiagen kit (Qiagen, Valencia, CA, USA) was used for all PCR experiments with an amplification protocol that consisted of an initial denaturation step at 96°C for 5 min followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min with a final extension at 72°C for 5 min using a PTC-200 DNA Engine Cycler (MJ Research Inc., Waltham, MA, USA).

Results

Bacterial isolation

Bacteria were not detected in the asymptomatic seed samples. Streaking with a sterile glass rod that had been rubbed against a symptomatic seed resulted in inconsistent recovery of bacteria. Placing diseased seed that had been washed in water on TSA plates yielded a confluent yellow bacterial growth around the seed. In contrast, bacteria were isolated (with fungi occasionally) from 10-fold serial dilution plating of seed wash from discolored seed. Colony morphology of the bacteria consistently isolated from diseased seed was mucoid with a smooth, entire periphery and a yellow pigmentation on TSA. A total of 16 isolates with the colony morphology consistently observed from separate bolls were picked and purified from plates that had been inoculated with seed wash.

Pathogenicity tests

Three isolates designated Sc1, Sc2 and Sc4 were used in all tests to determine the optimal inoculation method (i.e. technique and location on plant). The superficial inoculation on stigma scars, wall sutures, and scratch wounds on bracts, calyx and bolls did not result in disease development. Symptoms were not induced by any of the isolates from puncture wound inoculations into the bract or pedicel. Infections only occurred when bacterial injections breached the endocarp of the boll either through the carpel wall or a suture between carpel sections. Disease symptom development at 1 week postinoculation was limited to tissue surrounding the injection (*c.* 3 mm) with spotty salmon pink discoloration of the seed coat. In contrast, seed and lint discoloration occurred throughout the inoculated locule 2, 3 and 4 weeks postinoculation. Therefore, the deduced disease model involved infiltration of the bacteria through the capsule wall at the suture of one locule per immature boll using a needle, followed by the harvesting of the fruit 2 weeks postinoculation. Each of the 16 representative isolates tested by wound inoculation consistently resulted in disease and locule rot.

Incorporation of an antibiotic-resistance marker was employed to fulfil Koch's postulates and measure bacterial densities in inoculated fruit. Spontaneously generated Rif-resistant mutants were acquired for the isolates (Sc1, Sc2 and Sc4) determined to be capable of causing seed and boll rot. Rifampicin resistance for Sc1-R, Sc2-R and Sc4-R was stable for the 2-week testing period. There were no statistically significant differences (F was only significant at $P < 0{\cdot}01$) in growth patterns between the Rif-resistant mutants and their respective parental strains (data not shown).

The disease model was utilized to compare the infection potential of the Rif resistant and respective Rif-sensitive parental strains. Modifications of the technique included injecting bacteria with a syringe and 28G needle at a final concentration of 10^6 CFU ml^{-1}. Bacteria were not recovered from asymptomatic control locules (Fig. 1) that were injected with PO_4 buffer and plated on either selective or non-selective media 2 weeks postinoculation. Following the 2-week incubation period, locules inoculated with parental strains were consistently diseased with bacterial concentrations of 10^8 CFU g^{-1} tissue on TSA, whereas no bacteria were detected on parallel TSA plates amended with Rif. Disease symptoms and bacterial populations (10^8 CFU g^{-1}) in locules inoculated with the Rif mutants were comparable with those of parental strains. However, the Rif mutants were detected at equivalent levels on TSA with or without the antibiotic. Locules from bolls that were injected with PO_4 buffer 2 weeks postanthesis and then grown to maturity had scar tissue at the site of the initial inoculation (Fig. 1). Conversely, mature open bolls with locules that were inoculated with either the Rif-resistant mutants or the parental types developed necrosis of the entire locule. Discolored seed from rotten locules were cross-sectioned; embryos were shrivelled and brown in coloration. Regardless of whether parental strains or the Rif-resistant mutants were used, disease occurred only in locules that were injected with bacteria.

Disease development and bacterial populations in locules inoculated with Rif-resistant mutants or the respective parental types were determined to be comparable. Therefore, mutant Sc1-R was inoculated at a range of concentrations to examine potential effects of inoculum

Figure 1 Differences between control and diseased greenhouse grown fruit with a locule inoculated 2 weeks postanthesis using a 28G needle. (a) A locule injected with 10 μl of PO_4 buffer (pH 7·1) showing no apparent infection symptoms 2 weeks postinoculation. (b) A locule injected with strain Sc1-R showing disease symptoms 2 weeks postinoculation. (c) An open boll showing a locule injected with 10 μl of PO_4 buffer (pH 7·1) 2 weeks postanthesis with apparent scar tissue from the initial puncture wound. (d) An open boll showing a locule injected with strain Sc1-R 2 weeks postanthesis showing disease symptoms.

Table 2 Recovery of strain Sc1-R from locule tissue of inoculated greenhouse grown cotton fruit

Inoculation (CFU ml^{-1})	Boll appearance	Bacteria recovered (TSA) (CFU g^{-1})*	Bacteria recovered (TSA + Rif) (CFU g^{-1})*
Sc1-R†			
10^8	No exterior symptoms; entire locule infected and rotted	1×10^{10}	1×10^{10}
10^6	No exterior symptoms; entire locule infected and rotted	5×10^8	5×10^8
10^3	No exterior symptoms; entire locule infected and rotted	5×10^8	5×10^8
Sc1-R (heat killed)‡			
10^8	Puncture wound visible; no discoloration of locule	$<10^1$	$<10^1$
10^6	Puncture wound visible; no discoloration of locule	$<10^1$	$<10^1$
10^3	Puncture wound visible; no discoloration of locule	$<10^1$	$<10^1$
PO_4 buffer	Puncture wound visible; no discoloration of locule	$<10^1$	$<10^1$

TSA, trypticase soy agar; Rif, rifampicin.
*Bolls were harvested 2 weeks following inoculations and dilution plated on both TSA medium and TSA amended with Rif (200 μg ml^{-1}).
† Suspensions of bacteria in PO_4 buffer (pH 7·1) were injected using a 28G needle into a single locule per boll 2 weeks postanthesis.
‡Cells were exposed to 65°C for 1 h.

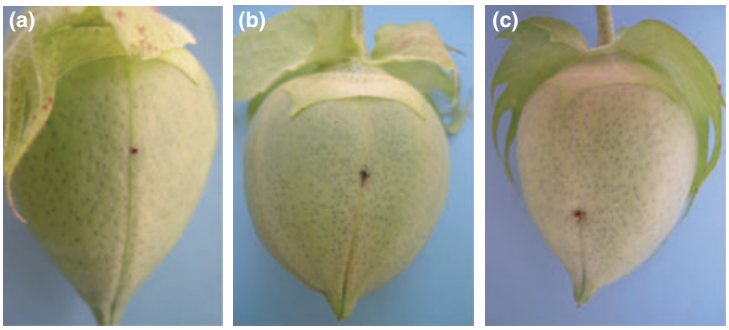

Figure 2 Outer carpel effects of greenhouse grown bolls injected 2 weeks postanthesis with (a) 10 μl of PO_4 buffer (pH 7·1); (b) strain Sc1-R (10^8 CFU ml^{-1}) or (c) Sc1-R heat-killed cells (10^8 CFU ml^{-1}) using a 28G needle then harvested 2 weeks post-inoculation.

on disease symptom expression and bacterial populations (Table 2). The outer carpel of bolls inoculated with Sc1-R had no apparent disease symptoms regardless of the bacterial concentration used (Fig. 2). Injections of Sc1-R at either 10^3 or 10^6 CFU ml^{-1} resulted in locule symptoms analogous to field infected boll samples with bacterial concentrations detected at 10^8 CFU g^{-1} 2 weeks following the inoculations (Table 2). Disease symptoms were contained to the inoculated locule 2 weeks after injecting bolls with 10^8 CFU ml^{-1} of the mutant. The locule tissue was completely shrivelled, and bacterial concentrations were measured at 10^{10} CFU g^{-1} of diseased tissue. In contrast, locules injected with either heat-killed cells or PO_4 buffer were asymptomatic (Table 2). Furthermore, no growth on TSA was detected from cell suspensions of either 10^3, 10^6 or 10^8 CFU ml^{-1} following exposure to heat-killed conditions.

Strain Sc1-R was utilized to study effects of boll age at inoculation on both the severity of rot symptoms and bacterial densities (Table 3). All bolls punctured <6 DPA were aborted, were cavitated or had split prematurely. Bolls inoculated with Sc1-R at 6–8 DPA had extensive disintegration of the locules leaving only a small black hard mummy or sooty black residue, and therefore, bacterial densities were not determined (Table 3). Control bolls 6–8 DPA were prone to fungal contamination. Locules from bolls inoculated 13–15 DPA with Sc1-R exhibited symptoms that resembled the infected field cotton bolls (Fig. 3) from which the Sc1 parental strain was isolated. Disease symptoms such as dark spots or stripes that developed immediately and contiguously under the suture were apparent throughout the inoculated locule. The lint was stained brown, decomposed and not viscous. The three non-inoculated locules from the same bolls showed no disease symptoms. Levels of Sc1-R in infected locules were consistent with densities determined from studies that compared the Rif mutants and the parental strains (10^8 CFU g^{-1}). Bolls inoculated at 20–28 DPA with derivative Sc1-R expressed symptoms that extended only to the area immediately surrounding the injection point. Bacterial densities from locules inoculated 20–28 DPA were detected at 10-fold lower levels than populations from bolls inoculated 13–15 DPA.

Phenotypic characterization, FA profiling and 16s ribosomal DNA sequencing of the pathogens

Isolates Sc1, Sc2 and Sc4 were rod-shaped, Gram-negative, facultative anaerobes and catalase positive. With the exception of the gelatinase test, the API 20E results for

Table 3 Disease symptoms resulting from wound inoculation of cotton cv. Acala Maxxa at different boll ages with strain Sc1-R

	Sc1-R*		Control†	
Boll age at inoculation	Boll appearance‡	Bacteria recovered§	Boll appearance	Bacteria recovered
6–8 days postanthesis	Rot of all locules; boll abscission	Not done	Discoloration at inoculation point (c. 1 mm)	Not done
13–15 days postanthesis	Entire locule infected and rotted	5×10^8 CFU g^{-1}	Discoloration at inoculation point (c. 1 mm)	$<10^1$ CFU g^{-1}
20–22 days postanthesis	Necrosis at inoculation point (c. 3 mm)	1×10^7 CFU g^{-1}	Discoloration at inoculation point (c. 1 mm)	$<10^1$ CFU g^{-1}
27–28 days postanthesis	Necrosis at inoculation point (c. 1 mm)	1×10^5 CFU g^{-1}	Discoloration at inoculation point (c. 1 mm)	$<10^1$ CFU g^{-1}

*A bacterial suspension in PO_4 buffer (pH 7·1) was inoculated through the capsule of a single locule at a final concentration of 10^6 CFU ml^{-1} using a 28G needle.
†Mock inoculations of bolls involved injecting PO_4 buffer (10 μl) into the capsule of a single locule using a 28G needle.
‡Bolls were harvested 14 days after the inoculation date.
§Samples were plated on trypticase soy agar amended with rifampicin (200 μg ml^{-1}).

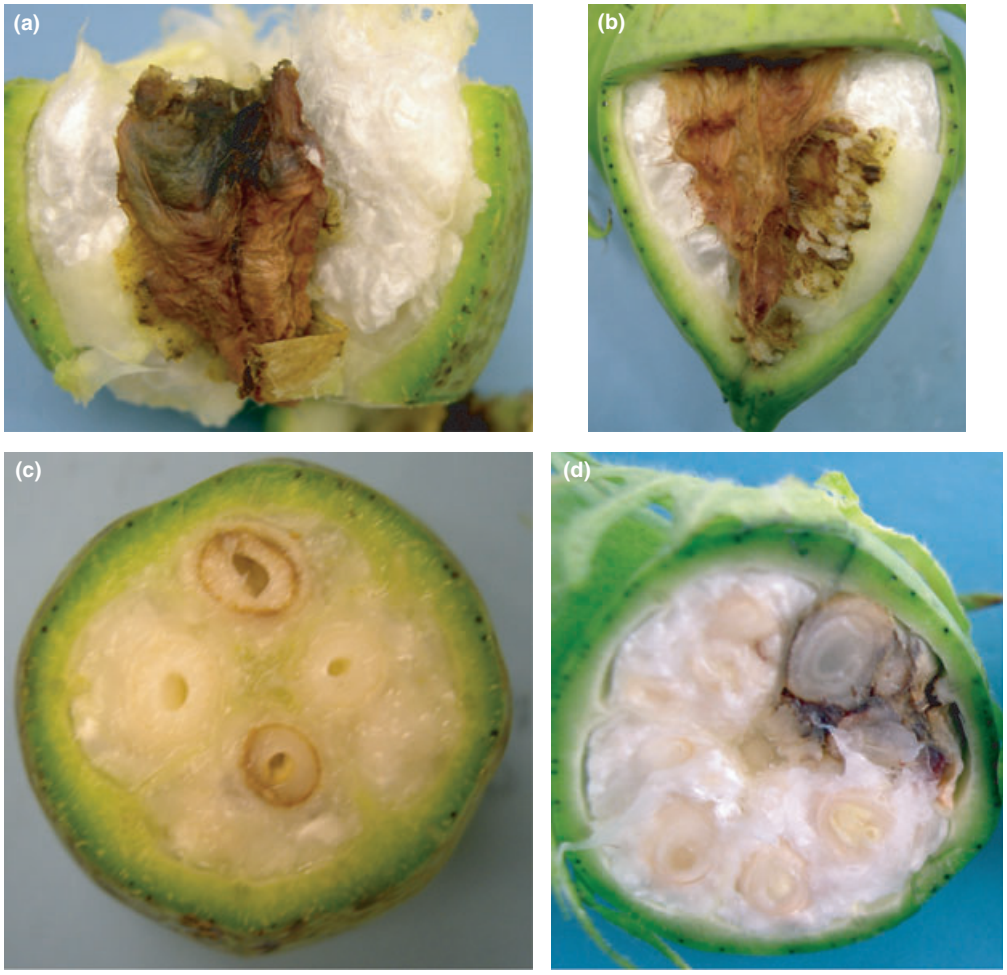

Figure 3 Symptom expression of diseased cotton fruit. (a, c) Field infected cotton boll samples collected in South Carolina. (b, d) Greenhouse grown fruit injected 2 weeks postanthesis with strain Sc1-R using a 28G needle then harvested 2 weeks postinoculation.

the three unknown isolates were identical to the reactions of the *P. agglomerans* type strain (Table 4). Isolate Sc2 and *P. agglomerans* ATCC 27155 did not liquefy the charcoal gelatin, whereas isolates Sc1 and Sc4 diffused the substrate after 24 h. The isolates (Sc1, Sc2 and Sc4) and the respective Rif-resistant mutants were identified as *P.*

Table 4 Phenotypic and biochemical characteristics of isolates Sc1, Sc2 and Sc4 that included the *Pantoea agglomerans* type strain ATCC 27155

Test	Sc1	Sc2	Sc4	*P. agglomerans*
Yellow pigment on TSA	+	+	+	+
Taupe pigment on YDC	–	–	–	–
Growth at 37°C	+	+	+	+
Motility	+	+	+	+
Production of*				
Beta-galactosidase	+	+	+	+
Arginine dihydrolase	–	–	–	–
Lysine decarboxylase	–	–	–	–
Ornithine decarboxylase	–	–	–	–
H_2S	–	–	–	–
Deaminase	–	–	–	–
Indole	–	–	–	–
Acetoin (Voges–Proskauer reaction)	+	+	+	+
Gelatinase	+	–	+	–
Citrate utilization*	–	–	–	–
Urea hydrolysis*	–	–	–	–
Acid production from*				
Glucose	+	+	+	+
Mannitol	+	+	+	+
Inositol	–	–	–	–
Sorbitol	–	–	–	–
Rhamnose	+	+	+	+
Sucrose	+	+	+	+
Melibiose	–	–	–	–
Amygdalin	+	+	+	+
Arabinose	+	+	+	+
Oxidase	–	–	–	–

TSA, trypticase soy agar; YDC, yeast extract-dextrose-$CaCO_3$.
*Testing results were determined using the API 20E system.

agglomerans based on a FA profile analysis with similarity indices of 0·94 for each isolate. A 1·5-kb portion of the 16s rDNA gene for isolates Sc1, Sc2 and Sc4 was cloned and sequenced. The percentage of identities between sequences from all of the tested isolates was 99% when compared to the 16s rDNA of *P. agglomerans* strain A80 (accession number AF130945).

Discussion

In this study, *P. agglomerans* was isolated from immature diseased bolls collected from a field in SC. Several of these isolates were used in experiments designed to determine whether the isolates were capable of causing disease in greenhouse grown bolls with symptoms analogous to infected field samples. Superficial inoculations of the bacteria on various parts of the fruiting body (i.e. stigma scars, wall sutures, bracts, calyxes and petioles) did not result in disease. In contrast, injection of the bacteria into locules of an immature boll at the suture resulted in the development of disease symptoms that noticeably resembled diseased fruit in the field (Fig. 3). Infection spread throughout the inoculated locule by 2 weeks postinoculation (Fig. 1). Diseased bolls grown to maturity revealed matted, mummified inoculated locules (Fig. 1) with lint and seed stained brown consistent with diseased field fruit.

Isolation of bacteria from immature diseased cotton seed has been reported in other work focused on determining the cause of SC seed rot (Hollis 2001). However, no correlation was detected between the bacteria found in seed at planting and the isolates recovered from diseased seeds in bolls suggesting that the opportunistic pathogens may have to be introduced into immature bolls. Inoculations of bacteria, isolated from disease samples, by injection into bolls 2 weeks postanthesis resulted in consistent disease development. Furthermore, the lack of disease development from superficial inoculations, and punctures of bracts or pedicels indicated that *P. agglomerans* requires a wound that penetrates the capsule for boll infection to occur. The infiltration requirement indicates that field infections occur via a vector even though macroscopic signs of insect feeding in diseased field samples are not always readily apparent (Willrich *et al.* 2004). Comparably, reductions in disease incidence have been correlated with the application of insecticides to pineapple fields with a history of pink disease (Kado 2003). However, the field infection process of pineapples by *Pantoea citrea* that causes pink disease has not been identified (Kado 2003).

Resistance to Rif was introduced into isolates putatively determined to cause disease for use as a selection marker in order to clearly differentiate between Rif-sensitive contaminants and the inoculated Rif-resistant mutants. Rifampicin has a broad bacterial host range with a mode of action that consists of irreversible binding to the β-subunit of RNA polymerase, thus inhibiting bacterial transcription (Campbell *et al.* 2001). Stability of Rif resistance in engineered mutants has also been exploited in root colonization field studies (Glandorf *et al.* 1992). In the current report, resistance to the antibiotic was determined to remain stable in Rif-resistant derivatives under nonselective conditions. Apparently, Rif resistance did not alter bacterial growth or the ability to infect greenhouse grown fruit when compared with the parental isolates. The selection marker provided a means to definitively conclude that the bacteria recovered from diseased bolls were the strains that were inoculated, thereby fulfilling Koch's postulates.

Determining that the infection potential of strain Sc1-R was not statistically different from the parental isolate justified the exclusive use of the mutant to examine effects of bacterial concentrations (Table 2) and boll age (Table 3) on both symptom development and bacterial densities in infected tissues. Symptoms that closely resembled those of field diseased bolls were produced in greenhouse grown

fruit by inoculating at the lowest concentration of Sc1-R tested (10^3 CFU ml^{-1}). However, the detected bacterial levels (10^8 CFU g^{-1} tissue) from bolls inoculated with 10^3 or 10^6 CFU ml^{-1} were 100-fold lower than in bolls injected with 10^8 CFU ml^{-1} (10^{10} CFU g^{-1} tissue). Differences between the numbers of recovered bacteria are likely due to the initial inoculation concentrations. Furthermore, the 2-week incubation period may not have been enough time for equivalent bacterial levels to be attained by the three inoculation concentrations tested.

The age of the boll at inoculation affected disease expression. Inoculations of bolls <2 weeks postanthesis resulted in complete rot and abscission of the boll despite the fact that only one locule was inoculated; this indicated possible bacterial movement (i.e. invasion) into non-inoculated locules and/or irrecoverable trauma inflicted by the needle puncture. Nevertheless, it should be noted that abiotic/biotic stresses (i.e. heat stress, cloudy weather, insect or mechanical injury) can result in abscission of young field grown bolls. Data from bolls inoculated 13–28 DPA suggested a containment of the infection that increased with boll maturity at the time of the challenge (Table 3). The highest bacterial densities were detected from locules injected with Sc1-R at 13–15 DPA (Table 3). Notably, the symptoms exhibited by these bolls closely resembled field infected fruits (Fig. 3) from which Sc1 was isolated. Seeds from locules inoculated at a maturity of 20–28 DPA appeared unaffected by the bacterial challenge based on coat coloration and embryo anatomy. Collectively, these results may provide insight to the level of susceptibility of developing bolls to this opportunist under field conditions.

A polyphasic taxonomic analysis that included phenotypic testing, FA profiling and 16s rDNA sequence comparisons all resulted in identification of the isolates as *P. agglomerans*. Although a bacterial marker was not employed, Ashworth *et al.* (1969) discussed the infection of immature bolls collected from fields in the California San Joaquin Valley by *Pantoea* spp. (synonym *Erwinia*; Gavini *et al.* 1989). Interestingly, the symptoms depicted in that study are basically identical to disease symptoms described both in SC field samples and the infected greenhouse fruits discussed in this communication. Ashworth *et al.* (1969) found no macroscopic injuries on the surface of the infected bolls; internal necrosis was noted. Moreover, the bacterium was reportedly transmitted by the brown stink bug (*Euschistus servus* S.).

Pantoea agglomerans has been traditionally regarded as a soil saprophyte (Gavini *et al.* 1989). Strains have since been found to inhabit both internal and external tissues of plants including cotton (Misaghi and Donndelinger 1990; McInroy and Kloepper 1995; Quadt-Hallman *et al.* 1996; Adams and Kloepper 2002; Sabaratnam and Beattie 2003). Strains of *P. agglomerans* can opportunistically infect plants triggering gall formations (Ezra *et al.* 2004) or human wounds causing septic arthritis (Kratz *et al.* 2003). In the current report, we determined that *P. agglomerans* infection of immature bolls of greenhouse grown cotton produced symptoms similar to diseased field samples observed in SC cotton. An inoculation method involving the injection of *P. agglomerans* in order for disease to occur suggested the involvement of a vector in field infections. Willrich *et al.* (2004) reported an increase in the incidence of hard locks in bolls exposed to southern green stink bugs (*Nezara viridula* L.) even though injury due to insect feeding was not always evident. Therefore, studies using laboratory reared *N. viridula* periodically provided food contaminated with strain Sc1-R and then caged with green bolls are in progress to determine the potential role of stink bugs in cotton boll and seed rot infections. Furthermore, bacterial isolations from diseased field samples are ongoing in order to study possible seasonal variations and a potential spectrum of opportunistic species.

Research focused on defining the genetics of *P. agglomerans* pathogenicity is being conducted with the objective of developing a PCR-based system for direct detection of the pathogen from field samples. Genes that encode for pathogenic determinants of *P. agglomerans* will be targeted. The system will eliminate the need for traditional culture and classification procedures that involve isolating and identifying *P. agglomerans* followed by disease testing to distinguish between saprophytic and pathogenic strains, an energy intensive and time-consuming process. The PCR-based detection method will be utilized in molecular epidemiological studies to test for the distribution of the boll rot pathogens in field grown cotton and/or surrounding vegetation that may serve as a reservoir.

Acknowledgements

Michael A. Jones collected bolls used for isolations from the Pee Dee Research and Education Center, Florence, SC. Thanks to Lacey Doughty, Laura Marie Kramer, Brittany Tiner, and Krista Doughty for their technical assistance. The API 20E testing and results analysis were performed by Kate Andrews.

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